## REMARKS

### THE AMENDMENTS

Applicants acknowledge, with appreciation, Examiner's indication that the rejection of claims 2-3, 8-9, 14 and 19 under 35 U.S.C. §103(a) and of claim 8 under 35 U.S.C. §112, second paragraph, made in the Office Action mailed on April 17, 2007 are withdrawn.

Applicants have amended Claim 9 to improve its form and has amended Claim 19 for clarity. Claims 2-3, 8-10, 14, and 19 are pending in the present application.

These amendments are made without prejudice and without waiver of applicants' right to pursue any canceled subject matter in one or more applications claiming priority herefrom under 35 U.S.C. §120.

These amendments add no new matter.

Applicants request reconsideration of the above-identified application, in view of the above amendments and the following remarks.

### REJECTIONS

#### 35 U.S.C. §112, Second Paragraph

Claim 9 is rejected under 35 U.S.C. § 112, second paragraph, as "indefinite." Office Action, page 3.

The Examiner asserts that it is indefinite for reciting the phrase "or preferably four." Applicants have amended claim 8 to remove the phrase "or preferably four," thereby obviating this rejection.

Accordingly, applicants request the Examiner withdraw this rejection.

Claim 19 is rejected under 35 U.S.C. § 112, second paragraph, as "indefinite." Office Action, page 3.

Specifically, the Examiner asserts that claim 19 is indefinite for not making grammatical sense in its recitation of the phrase "determining whether an offspring of an individual afflicted with a phakomatosis, wherein said phakomatosis is a tumor suppressor gene disease *has an increased risk of developing the tumor suppressor gene disease comprising...*" Applicants have amended claim 19 to insert a comma after the phrase "wherein said phakomatosis is a tumor suppressor gene disease_,_" thereby clarifying that this phrase modifies the term phakomatosis. As such, it is understood that further recitation of the phrase "the tumor suppressor gene disease" throughout the claim refers to the tumor suppressor gene disease which is a phakomatosis. Thus, as amended, the claim is clear and has consistent claim terminology and breadth throughout.

Accordingly, applicants request the Examiner withdraw this rejection of claim 19 and of claims dependent thereon (e.g., claims 2-3, 8-10, and 14).

35 U.S.C. §112, First Paragraph

Claims 2-3, 8-10, 14, and 19 are rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the enablement requirement.

The Office Action recites the Wands factors as criteria for establishing whether the disclosure would require undue experimentation and alleges that the claims lack enablement for "a method of determining whether an offspring of an individual afflicted with any type of phakomatosis..." Office Action, page 3.

The breadth of the claims & enablement issues:

The Office Action alleges that the "instant specification...does not reasonably convey to a skilled artisan that the method as claimed can be practiced fully commensurate in scope of the claimed breadth." The Office Action recites neurofibromatosis, tuberous slerosis, Sturge-Weber syndrome, and von Hippel-Lindau disease as specific examples of different phakomatosis diseases, asserting that the specification "fails to demonstrate that their method is also applicable to other conditions

embraced by the broad terminology, 'phakomatosis.'" Office Action, page 4.

Applicants believe that the Examiner may have misunderstood what is claimed. The claims as currently presented do not encompass "...any type of phakomatosis..." Rather, they are directed to a subset of phakomatoses. Specifically, the claims as currently presented are directed to the subset of phakomatoses that are tumor suppressor gene diseases. As presently amended (for form), claim 19 recites methods "for determining whether an offspring of an individual afflicted with a phakomatosis, *wherein said phakomatosis is a tumor suppressor gene disease*, has an increased risk of developing the tumor suppressor gene disease..." (Emphasis added.)

Applicants assert that the breadth of the claims as currently presented, specifically as related to phakomatoses which are tumor suppressor gene diseases, is fully enabled by the present disclosure.

The amount of direction or guidance present:

The Office Action asserts that the "...application's disclosure and guidance is directed solely to a single species of phakomatosis, neurofibromatosis," alleging that there is no "discussion

or guidance in the specification which would guide a skilled artisan to practice the invention drawn to other species embraced by the term, 'phakomatosis.'" Office Action, pages 4-5. Applicants disagree.

The language throughout the specification as originally filed clearly indicates that neurofibromatosis is merely an exemplary embodiment of the invention and not the full scope of the invention. In fact, the language used throughout the specification, as originally filed, is non-limiting and makes clear that the scope of the invention is broader than the any particular embodiment discussed therein. Furthermore, the application makes clear that the methods described for neurofibromatosis within the application are exemplary for all tumor suppressor gene disease phakomatoses.

For example, both the Field of the Invention and the Abstract state that "the invention concerns a method for the determination of data for the preparation of the presymptomatic or prenatal diagnosis of phakomatosis..." (Page 1, lines 4-6, and Abstract, lines 2-4.) Furthermore, the specification clearly states that "the problem [of the current state of the art for diagnosis of phakomatosis, in particular of tumor suppressor gene disease,] is solved

according to the invention by means of a method according to the claims." (Page 2, lines 17-18.) The claims as originally filed recite "a method for the determination of data for the preparation of presymptomatic or prenatal diagnosis of phakomatosis comprising the steps..." (Claims 1 and 4 as originally filed.) Accordingly, the methods recited in the claims as originally filed provide ample direction and clear guidance for the skilled artisan to practice the full scope of the presently claimed invention, e.g., for practice related to phakomatoses that are tumor suppressor gene diseases in general, not restricted to the exemplary embodiment species of neurofibromatosis.

The nature of the invention, unpredictability in the art, & state of prior art:

The Office Action describes the nature of the invention as being related to the "correlation of gene mutations," asserting that such is highly unpredictable in the art. (Office Action, page 5.) However, the nature of the invention, as unambiguously described throughout the specification, is related strictly to a method of determining whether the offspring of an individual afflicted with a tumor suppressor gene disease phakomatosis

has an increased risk of developing the tumor suppressor gene disease.

In describing the state of the prior art and the predictability or lack thereof in the art, the Office Action refers to the disclosures of Lucentini et al. (The Scientist, 2004, vol. 18), Wacholder et al. (J. Natl. Cancer Inst., 2004, 96(6), 435-442), and Ioannidis et al. (Nature Gen., 2001, 29, 306-309), all reporting discrepancies with correlation of gene mutations. However, none of these references specifically assesses the predictability of genetic analysis associated with phakomatoses which are tumor suppressor gene diseases. In fact, of the 36 different genetic disease associations detailed in Ioannidis et al., there is not one phakomatosis listed. Thus, the art cited in the Office Action is not directly applicable to the diseases relevant to the present invention. Furthermore, as stated above, the subject of the art cited, namely gene mutation analysis, is not the subject of the nature of the invention. More importantly, Applicants' invention is not the discovery of a correlation between a particular mutation and a disease. Rather, the invention involves detecting the presence or absence of an allele for determining whether the offspring of an

individual afflicted with a tumor suppressor gene disease phakomatosis has an increased risk of developing the tumor suppressor gene disease.

In fact, of the tumor suppressor gene disease phakomatoses listed in the Office Action (page 4), neurofibromatosis, tuberous sclerosis, and von Hippel-Lindau disease, the corresponding tumor suppressor genes had been identified by the time of applicants' filing date. The NF1 and NF2 genes of neurofibromatosis were identified, *inter alia*, in the Background of the Invention in the application as originally filed. (See, for example, page 2, lines 2-9.) The TSC2 and TSC1 genes of tuberous sclerosis are discussed, for example, in Carbonara, C., et al., Genes Chromosomes Cancer, 1996, 15(1), 18-25 (Exhibit A). The VHL gene of von Hippel-Lindau disease is discussed, for example, in Tory, K., et al., J. Natl. Cancer Inst., 1989, 81(14), 1097-1101 (Exhibit B). The well-established links between these tumor suppressor genes and their corresponding phakomatoses are evidence of the level of skill in the art associated with gene-disease correlation inside the area of phakomatosis.

The Office Action has also cited Tucker et al. (J. Natl. Cancer Inst., 2000, 92(7), 530-533) and its assertion of

the variation in the presence of loss of heterozygosity in various neurofibromas. Applicants respectfully submit that neurofibromas without loss of heterozygosity do not fall within the scope of the claimed invention. Claim 19, step d, clearly recites "...establishing the loss of an allele in the tumor of the afflicted individual, based on the comparison [of the amount and length of the one or more amplified polymorphous DNA microsatellite markers...]" As such, reference to this study is irrelevant and inapplicable to analysis of the present invention.

For at least these reasons, the scope of the present claims is fully supported by the specification. When the Wands factors are considered in light of the claims, the teaching of the present disclosure, and the level of skill in the art, it is clear that undue experimentation would not be required to practice the claimed invention. As such, the application, as filed, fully enables the claims as amended and the claims dependent thereon. Accordingly, applicants request the Examiner withdraw this rejection.

## Provisional Obviousness-Type Double Patenting

Claims 2, 3, 8-10, 14, and 19 stand provisionally rejected for nonstatutory obviousness-type double patenting as "unpatentable" over claims 1-16 of U.S. Patent No. 6,660,477, for reasons detailed in the August 15, 2006 Office Action.

Applicants stand ready to file a Terminal Disclaimer upon the Examiner's indication of allowable subject matter in this application.

## CONCLUSION

In view of the foregoing amendments and remarks, applicants respectfully request that the Examiner withdraw all the objections and rejections and allow all of the claims of this application.

Respectfully submitted,

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Exhibit A

GENES, CHROMOSOMES & CANCER 15:18–25 (1996)

# Apparent Preferential Loss of Heterozygosity at *TSC2* Over *TSC1* Chromosomal Region in Tuberous Sclerosis Hamartomas

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To investigate the molecular mechanisms of tuberous sclerosis (TSC) histopathologic lesions, we have tested for loss of heterozygosity the two *TSC* loci (*TSC1* and *TSC2*) and seven tumor suppressor gene-containing regions (*TP53*, *NF1*, *NF2*, *BRCA1*, *APC*, *VHL*, and *MLM*) in 20 hamartomas from 18 TSC patients. Overall, eight angiomyolipomas, eight giant cell astrocytomas, one cortical tuber, and three rhabdomyomas were analyzed. Loss of heterozygosity at either *TSC* locus was found in a large fraction of the informative patients, both sporadic (7/14) and familial (1/4). Interestingly, a statistically significant preponderance of loss of heterozygosity at *TSC2* was observed in the sporadic group ($P < 0.01$). Among the possible explanations considered, the bias in the selection for TSC patients with the most severe organ impairment seems particularly appealing. According to this view, a *TSC2* defect might confer a greater risk for early kidney failure or, possibly, a more rapid growth of a giant cell astrocytoma. None of the seven antioncogenes tested showed loss of heterozygosity, indicating that the loss of either *TSC* gene product may be sufficient to promote hamartomatous cell growth. Finally, the observation of loss of heterozygosity at different markers in an astrocytoma and in an angiomyolipoma from the same patient might suggest the multifocal origin of the second-hit mutation. *Genes Chromosom Cancer 15:18–25 (1996).* 

## INTRODUCTION

Tuberous sclerosis (TSC) is an autosomal dominant condition characterized by the presence of hamartomas in different organs, such as skin, brain, kidney, heart, retina, and lung (Gomez, 1991). Linkage analysis revealed genetic heterogeneity with two loci involved, *TSC1* and *TSC2*, on chromosome regions 9q34 and 16p13.3, respectively (Fryer et al., 1987; Northrup et al., 1987; Kandt et al., 1992). These two loci account for most multigenerational TSC families, whereas none of the other candidate gene regions has been clearly confirmed in more recent studies (Sampson and Harris, 1994). The ratio of *TSC1*- vs. *TSC2*-linked families appears to vary from 30% to 70% (Northrup et al., 1992; Kwiatkowski et al., 1993; Povey et al., 1994a). In the large majority (approximately two-thirds) of patients, the TSC appears to be sporadic (Sampson et al., 1989; Osborne et al., 1991). The number, size, and anatomic localization of the hamartomatous lesions show a dramatic individual variability, as documented by the long-lasting debate on TSC diagnostic criteria (Gomez, 1991; Roach et al., 1992). The reported TSC prevalence of 1/10,000 is likely to be underestimated, insofar as patients with very mild symptoms and no family history might go undiagnosed.

Recently, loss of heterozygosity (LOH) of microsatellite markers linked to 16p13.3 (Green et al., 1994a) or 9q34 (Carbonara et al., 1994; Green et al., 1994b) has been described for some TSC hamartomas, suggesting that both *TSC* genes could have a growth-suppressor-like function. To estimate whether *TSC1* and *TSC2* defects might have differential roles in TSC lesions of different organs and tissues, we have investigated for LOH both *TSC* loci as well as seven known tumor suppressor gene-containing regions in a series of angiomyolipomas, giant cell astrocytomas, and rhabdomyomas, and in one cortical tuber from 18 TSC patients.

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## MATERIALS AND METHODS

### Patients and Tissue Specimens

Twenty hamartomas (eight renal angiomyolipomas, eight giant cell astrocytomas, one cortical tuber, and three cardiac rhabdomyomas) obtained from 18 TSC patients were analyzed for loss of heterozygosity at *TSC1*, *TSC2*, and seven tumor suppressor gene loci. DNA extracted from peripheral blood leukocytes was available as control of the germline genotype in all cases except for Nos. 29, 59, 60, 67, and 68. Both maternal and paternal 16p13 and 9q34 haplotypes could be reconstructed from segregation analysis of the available family members (except for Nos. 67, 68, and 81). The diagnosis of TSC was made according to the criteria of Gomez (1991). Clinical examinations of the relatives of 10 probands revealed the presence of four familial cases (two *TSC1*-linked, Nos. 30 and 33; two compatible with both loci, Nos. 57 and 60). The family of patient 33 has been described previously (Carbonara et al., 1994). In the remaining eight families (Nos. 20, 26, 34, 58, 59, 67, 68, and 81), only anamnestic information about the relatives' clinical status was available. In two patients, lesions from two different pathologic sites could be analyzed (patient 3: giant cell astrocytoma and renal angiomyolipoma; patient 29: angiomyolipomas from the left and right kidneys).

### DNA Extraction and Polymerase Chain Reaction Analysis

DNA from 5 μm histologic sections of the paraffin-embedded hamartomas was extracted after a 48–72 hr incubation at 40°C with 200 μg/ml proteinase K in 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 100 μg/ml BSA, 0.45% Tween 20, and 0.45% Nonidet NP40 and a 10 min incubation at 94°C. DNA from peripheral blood leukocytes was extracted according to standard methods (Maniatis et al., 1989). Four to eight microliter aliquots were amplified in a 20 μl reaction containing 10 mM Tris, pH 8.3; 50 mM KCl; 200 μM each of dATP, dGTP, and dTTP; 20 μM dCTP; 0.1 μCi [$^{32}$P]αdCTP; 10 pmol of each primer; and 1 U Taq polymerase. Concentrations of $MgCl_2$ (0.75–2 mM) and annealing temperatures were optimized for each primer pair. Usually, 35–40 cycles were necessary for obtaining adequate products from biopsy specimens. The basic polymerase chain reaction (PCR) profile used was: 45 sec at 94°C, 45 sec at 48–65°C, and 1 min at 72°C, with a final extension for 5 min at 72°C. All PCR products were analyzed on a 6% denaturing polyacrylamide gel containing 25% formamide and 8 M urea.

The chromosome 16 markers tested were *HBAP1*, D16S525, D16S521, *KG8*, D16S291, D16S663, D16S283, D16S423, D16S407, D16S403, D16S401, D16S420, D16S296, D16S297, D16S299, D16S298, *SPN*, D16S409, D16S285, D16S416, D16S419, D16S514, and D16S402. On chromosome arm 9q, the *TSC1*-linked markers used, from centromere to telomere, were *ASS*, D9S125, D9S149, D9S150, D9S122, D9S66, D9S114, D9S67, and D9S158. Seven tumor suppressor gene-containing regions were investigated by means of the following polymorphic markers: *TP53*, *TP53CA* (Jones and Nakamura, 1992); *NF1*, 53.0 (Lazaro et al., 1993); *BRCA1*, D17S588; *APC*, D5S346; *VHL*, D3S1435; *NF2*, D22S268; *MLM*, *IFNA*, D9S171, D9S161, and D9S104. For further characterization of the 9p loss observed in one astrocytoma, the following markers have been included: D9S319, D9S52, D9S43, and D9S165. References for the microsatellite markers used can be found in the Genome Data Base (GDB).

## RESULTS

The analysis of *TSC1*- and *TSC2*-linked polymorphic markers in 20 hamartomas from 18 TSC patients is summarized in Figure 1. Eight 16p13.3- and nine 9q34-associated microsatellites were selected for genotyping of both the tumor and the control DNA, either from peripheral blood leukocytes or from normal tissue adjacent to the hamartoma. All patients were informative in at least one of the five loci spanning the *TSC1* critical region and at one or more loci both distal and proximal to *TSC2*.

LOH at two or more markers was a common finding, occurring in 10 of the 20 samples (50%); in particular, in five of eight angiomyolipomas and in five of eight giant cell astrocytomas. Interestingly, the 16p13 region was preferentially involved. LOH at 9q34 was present in only one case, a giant cell astrocytoma removed from a familial *TSC1*-linked patient (No. 33; previously described by Carbonara et al., 1994). No LOH could be documented in the second 9q34-linked familial case (rhabdomyoma No. 30) present in our sample collection.

If we do not take into account the four familial cases (Nos. 30, 33, 57, and 60), we are left with 14 patients who were informative at both loci. All of the 16p LOHs described here (nine samples from seven patients) were found within this subset, whereas no evidence of 9q LOH was obtained from

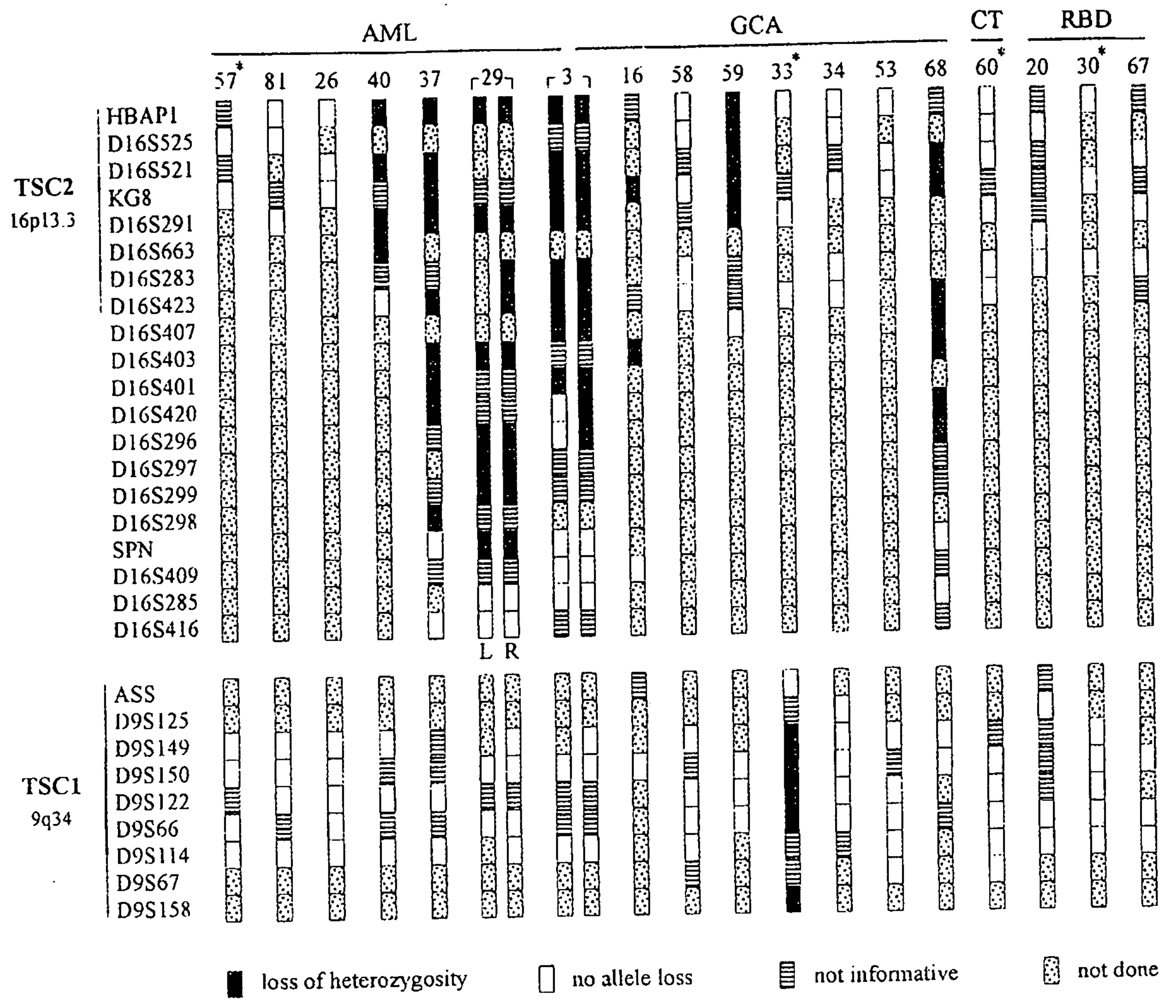


Figure 1. LOH analysis at the *TSC2* (upper panel) and *TSC1* (lower panel) loci in 20 hamartomas of 18 TSC patients, whose code numbers are given at the top. Two different lesions have been analyzed in patients 3 (angiomyolipoma and astrocytoma) and 29 [angiomyolipoma from the left (L) and right (R) kidneys]. The markers studied are indicated at left. AML, angiomyolipoma; GCA, giant cell astrocytoma; CT, cortical tuber; RBD, rhabdomyoma. The small solid rectangle at the left of the 16p13.3 markers indicates the position of the *TSC2* gene; the vertical black bar at the left of the 9q34 markers indicates the *TSC1* critical interval. The four familial cases are indicated by asterisks.

the same subjects. The parental origin of the chromosome 16 that had shown LOH could be established in six putatively sporadic patients; three were maternal and three paternal.

The physical range of the LOH phenomenon was assessed by means of additional microsatellites known to map at variable distances from *TSC1* and *TSC2*. In seven of the nine samples with LOH at chromosome 16 (four angiomyolipomas and three astrocytomas from patients Nos. 3, 16, 29, 37, and 68; see Fig. 1) the "deletion" appears quite large, spanning the 16p12 region. In at least three of these samples, the LOH extends to 16p11 markers as well (*SPN*, in the two angiomyolipomas of patient No. 29; D16S298, in the angiomyolipoma of patient No. 37). In the remaining two cases of 16p LOH (Nos. 40 and 59), the "deletion" is shorter, ending at D16S423 (16p13.3) and D16S407 (16p13.1-p13.2), respectively (Fig. 1). The 9q LOH (No. 33) is restricted to 9q34-qter. The most telomeric markers tested, *HBAP1* and D9S158, appear to be involved in all cases of LOH.

Two different lesions from two patients (Nos. 3 and 29) were studied in detail. As is shown in Figure 2, the two angiomyolipomas from the right and left kidneys of patient No. 29 have similar microsatellite patterns. In both lesions, the LOH breakpoint lies within a 1.7 cM interval, between *SPN* and D16S285. The LOHs in the giant cell astrocytoma and the renal angiomyolipoma of patient

No. 3 span different markers: D16S420 and D16S296 show LOH in the astrocytoma but not in the angiomyolipoma (Figs. 2, 3). Indeed, the LOH breakpoints differ in the two lesions, mapping between D16S296 and *SPN* in the astrocytoma and between D16S401 and D16S420 in the angiomyolipoma. The minimal genetic distance between the two LOH breakpoints is approximately 6 cM, i.e., equal to the D16S420-D16S296 interval.

It is worth noting that, in the angiomyolipomas, at variance with the astrocytomas, the "lost" allele often shows a residual, faint signal on autoradiography (Fig. 3). This phenomenon is likely to be due to the fact that angiomyolipomas are often intermingled with trace amounts of normal tissue and/or mononuclear cell infiltrates. Therefore, a consistent 50% or higher reduction in band intensity, compared to the pattern of control DNA, was considered indicative of LOH (see *HBAP1*, *KG8*, and D16S401 in Figure 3 as representative examples). Further evidence that the fainter alleles at multiple markers shown by angiomyolipoma No. 3 were consistent with a true LOH is that all faint 16p13-linked alleles could be assigned to the same parental haplotype.

Seven tumor suppressor gene-containing regions (*VHL*, *APC*, *MLM*, *TP53*, *NF1*, *BRCA1*, and *NF2*), located in five different chromosome segments (3p25, 5q21, 9p21, 17p13, 17q11, 17q21, and 22q12), were tested for LOH in 19 of the 20 hamartomas (Fig. 4). None showed evidence of LOH. Only the D9S104 marker appeared to have lost one allele (the paternal one) in astrocytoma No. 33 (Carbonara et al., 1994). All informative loci tested, both distal (D9S171, D9S161) and proximal (D9S43, D9S52, D9S319, D9S165) to D9S104, retained the germline configuration (not shown). Finally, no LOH at D9S104 was present in the other 12 hamartomas that were informative at this locus (Fig. 4).

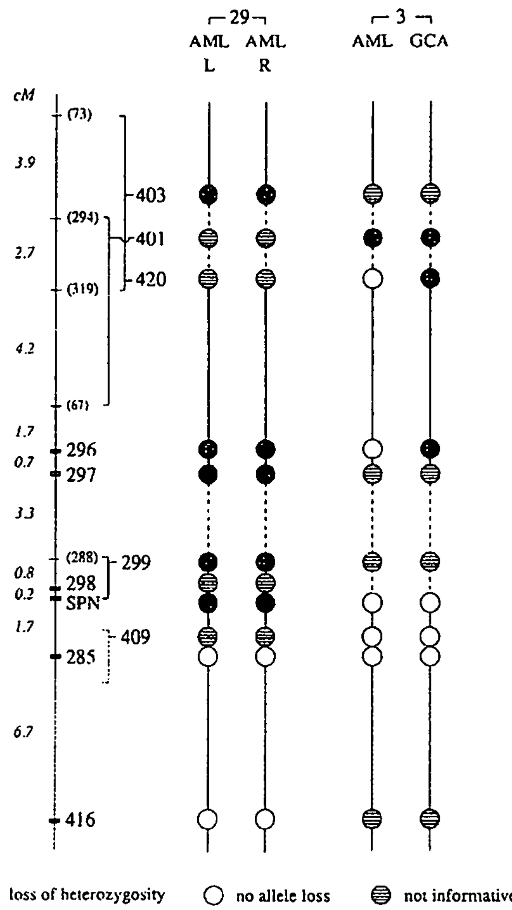


Figure 2. LOH analysis at 16p and 16q markers in the left (L) and right (R) renal angiomyolipomas (AML) of patient 29 and in the angiomyolipoma and giant cell astrocytoma (GCA) of patient 3. Genetic distances in centimorgans (cM) between tested loci or reference markers (in parenthesis) are according to the CEPH consortium linkage map of chromosome 16 (Kozman et al., 1995). D16S409 has been mapped according to Gyapay et al. (1994). Loci imprecisely mapped are separated by dashed lines.

## DISCUSSION

The pathogenetic mechanisms for the development of multiple hamartomas in TSC are still obscure. Important breakthroughs have been the mapping of two genes, *TSC1* and *TSC2*, at 9q34 (Fryer et al., 1987; Northrup et al., 1987) and 16p13 (Kandt et al., 1992), respectively, which account for most, if not all, familial cases (Sampson and Harris, 1994), and the observation of LOH at either region in some TSC hamartomas (Green et al., 1994a,b; Carbonara et al., 1994). On the basis of the latter findings and the GAP-like putative function of the recently cloned *TSC2* gene (European Chromosome 16 Tuberous Sclerosis Consortium, 1993), it has been suggested that the "two-hit" mutation model, already confirmed in more than a dozen tumor suppressor loci (Knudson, 1993), might fit TSC as well. According to this model, a cell should require biallelic loss-of-function mutations at either *TSC* locus in order to become the progenitor of hamartomas; in a TSC patient, the first inactivating mutation is likely to be germline, whereas the second allele is knocked out somatically. Open questions are whether the second hit is uni- or multifocal in origin, at which developmental stage it occurs, whether *TSC1* and *TSC2* defects show some sort of tissue specificity or different clinicopathologic severity, and finally

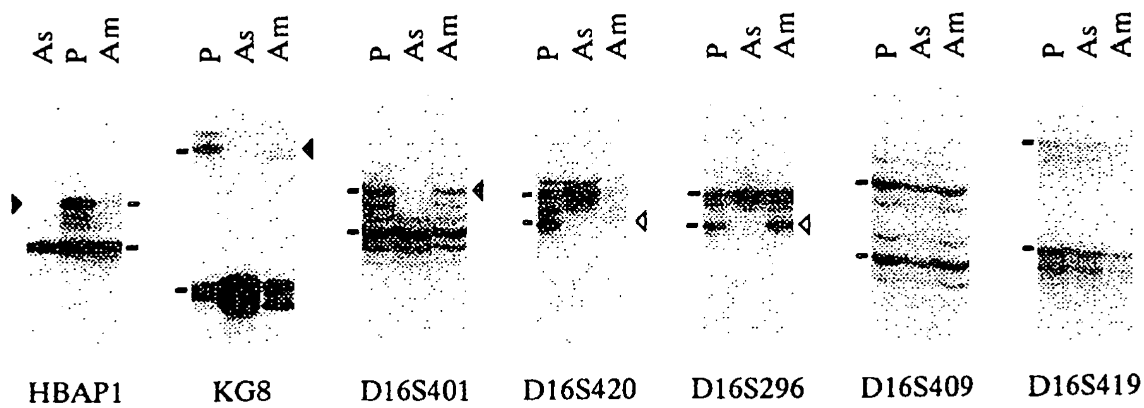


Figure 3. Polyacrylamide gel electrophoresis analysis of *HBAP1*, *KG8*, D16S401, D16S420, D16S296, D16S409, and D16S419 genotypes from peripheral blood leukocytes (P), astrocytoma (As), and angiomyolipoma (Am) of patient 3. The solid arrowhead shows the allele lost in both the angiomyolipoma and the astrocytoma; the open arrowhead shows the allele lost in the astrocytoma only.

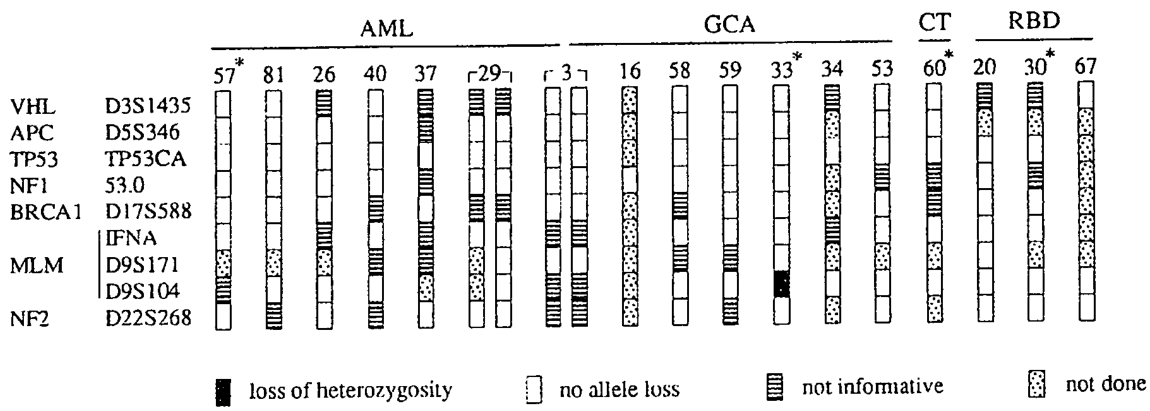


Figure 4. LOH analysis at seven tumor suppressor-containing gene regions. *VHL*, von Hippel-Lindau; *APC*, adenomatous polyposis coli; *NF1*, neurofibromatosis type 1; *BRCA1*, breast cancer gene 1; *MLM*, malignant melanoma; *NF2*, neurofibromatosis type 2. See the legend to Figure 1 for other details on the symbols used.

whether known antioncogenes are involved in the progression of the hamartomas.

In the present work, we have addressed these points through a detailed investigation of loss of heterozygosity at the two *TSC* loci and seven known tumor suppressor gene-containing regions on 20 surgical specimens of hamartomas removed from the kidney, brain, and heart of 18 TSC patients. The parents and other first-degree relatives of 10 probands, including seven of the eight showing LOH, were examined clinically, and four familial cases were found. The remaining eight probands, whose relatives could not be examined, had no family history of signs and symptoms of TSC. For simplicity, they were provisionally included among the sporadic group, although we are aware of the limits of such a classification because of the well-known variability of expression of the disease (Northrup et al., 1993).

Our data show that LOH at either *TSC* locus can be detected in a large fraction of the hamartomas (10/20; 50%), both from sporadic (7/14) and from familial (1/4) cases. The involvement of the two loci appears mutually exclusive; i.e., concomitant LOH at the two *TSC* loci was never found. Interestingly, a clear predominance of LOH at the *TSC2* locus is evident among the sporadic patients: In this subset, all documented *TSC* LOH occurred at 16p, i.e., in seven of 14 subjects informative at both loci, whereas no allele loss at 9q34 was found ($P < 0.01$). LOH at 16p13.3 and not at 9q34 has recently been reported in hamartomas from five of eight TSC patients (Green et al., 1994a). A series of possible explanations must be considered.

First, the LOH method might not be suitable for detection of second-hit mutations of *TSC1*, perhaps because large LOH regions are underrepresented at this locus, with microdeletions, point mutations, or gene conversions favored, whereas the majority of *TSC2* losses described herein are quite large, spanning the telomeric to the pericentromeric 16p markers. It is possible that, during ontogeny, a 9q LOH undergoes a higher negative selection than does an equivalent LOH at 16p, e.g., because of the higher gene content at 9q or because of parental imprinting of 9q-linked genes. The imprecise location of *TSC1* and the fact that not all subjects were informative at all loci spanning the 9q34 critical region might have contributed in part to the low rate of 9q-LOH detection.

A second possibility is that of a higher mutation rate of the *TSC2* gene. If this is indeed the case, a higher proportion of *TSC2*- vs. *TSC1*-linked families should be observed at the population level. Contradictory data on the prevalence of *TSC1*- vs. *TSC2*-linked families (30% to 70%) can be found in the literature (Northrup et al., 1992; Kwiatkowski et al., 1993; Povey et al., 1994a).

Intrigued by these findings, we thought that an ascertainment bias might be responsible for both the preferential *TSC2* involvement in the hamartomas and the preponderance of either *TSC1*- or *TSC2*-linked families in different studies. Multiple hamartomas are the characteristic signs of TSC, but, because they do not metastasize, their clinical consequences may vary from purely aesthetic ones, when the skin is mostly affected, to severe organ malfunction due to distortion of the architecture of a given tissue. Only occasionally, prompt removal of the cell mass is required, after the rupture of a large renal cyst, acute endocranial hypertension, or severe impairment of intracardiac blood flow. The subjects investigated in this study belong to the latter group, i.e., those with the most severe organ impairment. We therefore suggest that a *TSC2* germline mutation might confer a greater susceptibility to a rapid growth of angiomyolipomas and, possibly, giant cell astrocytomas.

Thus far, no statistically significant difference in dermatologic and/or neurologic signs and symptoms between patients with *TSC1*- and *TSC2*-linked disease has been confirmed (Povey et al., 1994b). Nevertheless, a quantitative estimate of the severity of kidney involvement in the two subsets of patients has not been attempted. It is therefore conceivable that differences in the severity of the probands' signs and symptoms account for the discrepancies in the reported prevalence of *TSC1*- vs. *TSC2*-linked families. A careful clinical and molecular investigation of a larger set of patients is necessary for verification of this hypothesis, which has important prognostic implications.

A second issue we have addressed is that of the uni- or multifocal origin of the somatic mutation. Hamartomatous lesions from both kidneys, or brain and kidney, were available from two TSC patients who showed LOH at 16p. Interestingly, in the giant cell astrocytoma and renal angiomyolipoma from the first patient (No. 3), the LOH breakpoints map to different genetic intervals, at least 5.9 cM apart (see Fig. 2). This finding suggests that two independent, postzygotic events might be responsible for the loss of the putatively normal *TSC2* allele in the two lesions. Whether they both occurred in a short temporal window of embryonic development, e.g., either before or some time after gastrulation, cannot be determined from the present data. To answer this question, a larger sample of affected tissues of both neuroectoderm and mesoderm origin should be tested in the same patient. Homozygosity for the *Tsc2* mutation appears to be lethal in the Eker rat (Kobayashi et al., 1995); if the early occurrence of the second-hit mutation is a general phenomenon in TSC, a dramatic cell selection would be expected to occur, occasionally interfering with the viability of the embryo. According to this view, one may predict that the isolated hamartomas often observed in subjects not fulfilling the minimal diagnostic criteria for TSC might reflect the later stage of embryonic development in which the inactivation of the second *TSC* allele took place.

An identical LOH pattern appeared in the two angiomyolipomas from the right and left kidneys of the second patient (No. 29). An apparently identical LOH region has been recently reported by Green et al. (1994a) from both a rhabdomyoma and a cortical tuber from a TSC patient. In our case, the two LOH breakpoints lie within the same 1.7 cM interval, in the 16p11 region. Because in at least five of the nine 16p LOHs described here the proximal breakpoints are located near or within this chromosomal region (16p12-p11), the latter finding is compatible either with a unifocal origin or with two independent events resulting in an apparently identical LOH pattern.

Finally, none of the seven tumor suppressor gene-containing regions tested showed clear evidence of LOH. Only the D9S104 marker, located at 9p21, a region frequently lost in many types of tumors (Diaz et al., 1988; Olopade et al., 1992, 1993; Cheng et al., 1993; Merlo et al., 1994),

showed LOH in a single lesion (a giant cell astrocytoma) among the 13 informative samples investigated. The recently identified gene for the inhibitor of the cyclin-dependent kinase 4 (CDK4I; Kamb et al., 1994; Nobori et al., 1994) was ruled out, because it lies telomeric to D9S104, between the *IFNA* and D9S171 markers, which both retain the germline configuration. We could narrow down the 9p LOH to a short interval: proximal to D9S161 and distal to D9S43, D9S52, and D9S319. This finding, although of uncertain significance for the pathogenesis of TSC hamartomas, might prove helpful for localization of other tumor suppressor genes near the MLM critical region. The apparent germline configuration at known antioncogene loci is not surprising, in that TSC is not a cancer-prone disease. Nevertheless, at present we cannot exclude the possibility that other genes play some role in the progression of the hamartomas. Although the functional inactivation of *TSC1* and *TSC2* appears to fit to the two-hit model originally described for the antioncogenes, it seems to us that terms such as stem cell growth suppressors or regulators may be more appropriate to the putative function of the *TSC* genes.

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# Specific Genetic Change in Tumors Associated With von Hippel-Lindau Disease

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**Previous reports showed that the loss of DNA sequences on the short arm of chromosome 3 (3p) is consistently found in sporadic renal cell carcinomas. To evaluate the significance of this genetic change, we looked for the loss of 3p alleles in hereditary renal cell carcinomas and other tumors from patients with von Hippel-Lindau disease. Specific loss of alleles from chromosome 3p was detected with polymorphic DNA markers in 11 renal cell carcinomas, one pheochromocytoma, two spinal hemangioblastomas and one cerebellar hemangioblastoma from von Hippel-Lindau patients. Multiple renal cell carcinomas in individuals with von Hippel-Lindau disease showed loss of the same chromosome 3p alleles, which demonstrated that the same chromosome was deleted in each tumor. Analysis of haplotypes indicated that the loss of chromosome 3p alleles was from the chromosome bearing the balancing, wild-type allele of the VHL gene. These results are consistent with the concept that the VHL gene is a recessive oncogene. Renal cell carcinoma, pheochromocytoma, and spinal and cerebellar hemangioblastomas develop in predisposed family members when somatic mutational events lead to loss of chromosome 3p sequences bearing the wild-type allele of the VHL gene. [J Natl Cancer Inst 81:1097-1101, 1989]**

von Hippel-Lindau disease is an autosomal dominant trait characterized by retinal angiomas, cerebellar and spinal hemangioblastomas, pheochromocytomas, renal cell carcinomas, and benign tumors of the testicle and pancreas (*1*). The VHL gene is linked to RAF1 (*2*), a gene located on chromosome 3p (*3*). The mechanism by which the VHL gene predisposes to neoplasia in different target organs is not known. One possibility is that the VHL gene is a recessive oncogene. Genes that lead to cancer when both copies have been inactivated by mutation have been referred to as recessive oncogenes or tumor suppressor genes (*4*). Retinoblastoma, Wilms' tumor, multiple endocrine neoplasia type 1, and bilateral acoustic neurofibromatosis (*5-9*) are among the malignant diseases in which recessive oncogenes play a critical role in tumor origin. The hallmark of a recessive oncogene is the specific and consistent loss of the wild-type allele of the disease gene in tumors of patients with heritable forms of cancer. In the absence of a probe for the disease gene, loss of the wild-type allele can be tested indirectly with polymorphic DNA markers located around the disease gene locus.

We performed an analysis of DNA polymorphism on 17 tumors obtained from seven patients with von Hippel-Lindau disease. Deletion of DNA sequences on chromosome 3p was detected in 11 renal cell carcinomas, one pheochromocytoma, two spinal hemangioblastomas, and one cerebellar hemangioblastoma. We could determine maternal or paternal origin of 3p alleles for six tumors; in all six, deletion of chromosome 3p DNA sequences was from the chromosome bearing the wild-type allele of the VHL gene.

## Methods

Tumor and non-neoplastic tissues were obtained from patients treated at The Johns Hopkins Hospital (Baltimore, MD), the Methodist Hospital (Houston, TX), and the Surgery Branch and Surgical Neurology Branch of the National Cancer Institute. The diagnosis of von Hippel-Lindau disease was based on established criteria (*1*). Each tumor was examined by the cryostat section technique before extraction of high-molecular-weight DNA. Conditions for digestion of DNA with restriction endonucleases, agarose gel electrophoresis, transfer to nylon membranes, and hybridization have been described (*10*).

To detect the somatic loss of chromosome 3p sequences, we typed tumor and corresponding normal (leukocyte or normal kidney) DNA with eight polymorphic DNA markers for chromosome 3: (*a*) D3S4, DNF15S2, and D3S32 are anonymous loci, members of a linkage group on chromosome 3p (*11*); the loci are ordered with D3S4 closest to the centromere (Nakamura Y, White R: unpublished data). D3S4 is detected by probe B67 (*12*); DNF15S2 is detected by probe pH3H2 and has been mapped to 3p21 (*13*). D3S32 is detected by probe pEFD145.1 (*11*). (*b*) ERBAβ, the avian erythroblastic leukemia viral oncogene homolog 2 detected by probe pBH302, has been mapped to 3p21-25 (*14,15*); RAF1, the murine leukemia viral oncogene ho-

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molog 1, has been mapped to 3p24-25 (*3*). (*c*) D3S17 and D3S18 are anonymous loci, members of a linkage group on 3p. D3S17 is detected by probe CRI-L892; D3S18 is detected by probe CRI-L162 (*16*). (*d*) D3S42, an anonymous DNA locus detected by probe pEFD64.1, has been mapped to chromosome 3q (Nakamura Y, White R: unpublished data). Six of seven patients with von Hippel-Lindau disease were heterozygous in their normal tissue for at least one of the eight polymorphic DNA markers, and we were therefore able to determine whether loss of constitutional heterozygosity had occurred in the respective tumor tissue.

To quantitate the loss of 3p alleles in tumor tissue, we measured the hybridization signals by scanning autoradiographs with a densitometer. We analyzed the data to determine the ratio of hybridization signals from constant bands from chromosome 1 (probe pH3H2) in normal tissue and tumor DNA and then used this factor to correct for differences in the amount of DNA in the lanes.

To determine whether the chromosome 3p alleles deleted in tumors of patients with von Hippel-Lindau disease were inherited from the affected or nonaffected parent, we determined the chromosome 3p genotypes of parents of patients with von Hippel-Lindau disease. We found that we could unambiguously assign the source of the deleted 3p allele in patients 1, 3, and 7 in whom the following criteria were met: (*a*) sample was obtained for DNA analysis from affected and nonaffected parents of the patient; and (*b*) one parent was heterozygous and the other was homozygous at the locus shown to be deleted in the tumor of the patient.

## Results

We examined renal cell carcinoma associated with von Hippel-Lindau disease for chromosome 3p allele loss because of the location of the VHL gene, reports of chromosome 3p alterations in two tumors of this type (*17,18*), and previous findings of consistent 3p allele loss in sporadic renal cell carcinoma (*19,20*). Examination of renal cell carcinomas from patients with von Hippel-Lindau disease may resolve the issue of whether chromosome 3p loss in renal tumors reflects a genetic change important in the origin or evolution of these tumors. We analyzed 11 renal cell carcinomas from three patients with von Hippel-Lindau disease; all (11 of 11) renal cell carcinomas had a loss of chromosome 3p alleles. There was a pattern to 3p allele loss in hereditary renal cell carcinomas. All four renal cell carcinomas from patient 1 had lost the same chromosome 3p allele (DNF15S2, allele 1) as shown in tables 1 and 2 and figure 1. Five of six renal cell carcinomas from patient 2 had lost the same chromosome 3p allele (DNF15S2, allele 1) depicted in tables 1 and 2 and figure 1. Analysis of segregation of chromosome 3p alleles in the families of patients 1 and 7 indicated that the chromosome that sustained the loss was inherited from the nonaffected parent (table 3).

Loss of chromosome 3p alleles in primary renal cell carcinomas was detected by reduction of allele signal intensity. With the probe for DNF15S2 the results were: in patient 1, allele 1 was decreased 57% in RCC1, 67% in RCC2, 60% in RCC3, and 52% in RCC4; in patient 2, allele 1 was decreased 52% in RCC1, 54% in RCC2, 43% in RCC3, 20% in RCC4, 75% in RCC5, and 0% in RCC6. The residual

**Table. 1** Chromosome 3p allele loss in tumors from patients with von Hippel-Lindau disease*

| Patient No. | Tumor | 3q | | Locus 3p | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | D3S42 (T) | D3S42 (P) | D3S4 (T) | DNF15S2 (H) | D3S32 (T) | ERBAβ (H) | RAF1 (B) | RAF1 (T) | D3S18 (T) | D3S17 (T) |
| 1 | RCC1 | 12 | | 12 | $\underline{2}$ | — | — | | | — | |
| | RCC2 | 12 | | 12 | $\underline{2}$ | — | — | 2 | | — | 2 |
| | RCC3 | 12 | | 12 | $\underline{2}$ | — | — | 2 | | — | 2 |
| | RCC4 | 12 | | 12 | $\underline{2}$ | — | — | | | — | |
| 2 | RCC1 | | 12 | 2 | 2 | — | — | — | | 2 | — |
| | RCC2 | | | | 2 | — | — | — | | | — |
| | RCC3 | | 2 | 2 | 2 | — | — | — | | 2 | — |
| | RCC4 | | | 12 | 2 | — | — | — | | | — |
| | RCC5 | | 2 | 2 | 2 | — | — | — | | 2 | — |
| | RCC6 | | 12 | 12 | 12 | — | — | — | | 2 | — |
| 3 | SH | — | | 12 | — | $\underline{12}$ | $\underline{12}$ | — | | 12 | — |
| | P | — | | 2 | — | $\underline{1}$ | $\underline{1}$ | — | | 2 | — |
| 4 | SH | 12 | | — | — | — | — | | | | |
| 5 | SH | 12 | | 12 | — | 2 | 12 | | 1 | | 2 |
| | CH | 2 | | 1 | — | 2 | 1 | | 1 | | 2 |
| 6 | SH | — | | 12 | 1 | 2 | 2 | | | | — |
| 7 | RCC1 | | | — | — | $\underline{1}$ | — | | | — | — |

*Loss of alleles at loci on chromosome 3p in different tumor types from patients with von Hippel-Lindau disease. Letters in parentheses indicate the restriction endonuclease: T, *Taq*I; M, *Msp*I; H, *Hin*dIII; B, *Bgl*I; P, *Pvu*II. Tumor phenotype is always shown when the blood leukocytes (or normal kidney) showed heterozygosity: "12" indicates heterozygosity in the tumor sample; "1" indicates retention of the larger allele and loss of the smaller allele; "2" indicates retention of the smaller allele and loss of the larger allele. Dash indicates that the sample was homozygous at that locus. Absence of an entry indicates that the sample was not tested because of an insufficient amount of DNA. A number "1" or "2" with an underline indicates a chromosome 3p allele was retained in the tumor and inherited from the parent affected with von Hippel-Lindau disease. RCC = renal cell carcinoma.

**Table 2.** Change in DNF15S2 alleles in renal cell carcinomas from patients with von Hippel-Lindau disease*

| Patient No. | Tumor | Allele ratio | Percent decrease in allele 1 |
|---|---|---|---|
| 1 | RCC1 | 0.39 | 57 |
| | RCC2 | 0.30 | 67 |
| | RCC3 | 0.67 | 60 |
| | RCC4 | 0.65 | 52 |
| 2 | RCC1 | 0.20 | 52 |
| | RCC2 | 0.25 | 54 |
| | RCC3 | 0.53 | 43 |
| | RCC4 | 0.52 | 20 |
| | RCC5 | 0.32 | 75 |
| | RCC6 | 0.93 | 0 |

*Changes in signal intensity (*19*) and ratio of signal intensity (*21*) were measured by quantitative densitometry in a previous study. Percent decrease in nine sporadic renal cell carcinomas varied from 29% to 60% (*19*). Ratio of larger and smaller DNA fragments (DNF15S2) in DNA extracted from normal kidneys was 1.082, with a 95% upper tolerance limit of 1.363 and a lower tolerance limit of 0.801. Value of allele ratio fell outside the 95% tolerance limits in patient 1 for renal cell carcinoma 1-4 (RCC1-RCC4); in patient 2, value for allele ratio fell outside the 95% tolerance limits for RCC1-RCC5.

signal present in renal tumor samples probably originated from host leukocytes known to be present in primary renal cell carcinomas (*19,22,23*). The hybridization signal of the remaining 3p allele was unchanged in tumors of patients 1 and 2, which indicates a deletion or total chromosome loss rather than loss and reduplication or mitotic recombination.

We tested renal cell carcinomas with a battery of eight probes previously mapped to chromosome 3 (table 1) to estimate the size of the deletion and obtain information on location of the VHL locus. In patient 1, the same chromosome 3p alleles were lost in each of the four renal cell carcinomas; the pattern of allele loss was consistent with a terminal deletion of chromosome 3p beginning distal to the D3S4 locus. However, the pattern of allele loss differed in the individual renal cell carcinomas from patient 2. That RCC3 and RCC5 had a loss of alleles at loci on 3p (D3S4, D3S30, DNF15S2) and 3q (D3S42) suggests a loss of the entire chromosome 3; a loss of alleles from RCC1 at three loci (D3S4, D3S30, DNF15S2) on chromosome 3p suggests an interstitial or a terminal deletion. RCC6 retained heterozygosity at the D3S4, D3S30, and DNF15S2 loci but had allele loss at the D3S18 locus. This result indicates that the VHL gene may be located distal to DNF15S2.

We analyzed two other histologic types of tumors associated with von Hippel-Lindau disease for chromosome 3p allele loss. Loss of alleles at loci on chromosome 3p (D3S32; ERBA$\beta$) was detected in a pheochromocytoma from patient 3 and in hemangioblastomas from patients 5 and 6 (table 1, fig. 2). Reductions in signal intensity of one allele at the D3S32 locus (pEFD145.1) in tumor tissue were quantitated by densitometry. In the pheochromocytoma of patient 3, the decrease was 56%; in the spinal and cerebellar hemangioblastomas of patient 5, the decreases were 49% and 85%, respectively; and in the spinal hemangioblastoma of patient 6, the decrease was 51%. The residual signal in the pheochromocytoma and hemangioblastomas probably originated from leukocytes and connective tissue cells present in the tumors. Haplotype analysis showed that the chromosome 3p alleles that were lost in the pheochromocytoma of patient 3 were on the chromosome inherited from the nonaffected parent.

The specificity of chromosome 3p losses was examined in nine tumors with recombinant DNA probes to loci located on nine other chromosomes (table 4). Heterozygosity was maintained in tumor tissue in 62 of 65 instances in which normal tissue was heterozygous at the tested locus. One of four renal cell carcinomas from patient 1 and the pheochromocytoma from patient 3 had

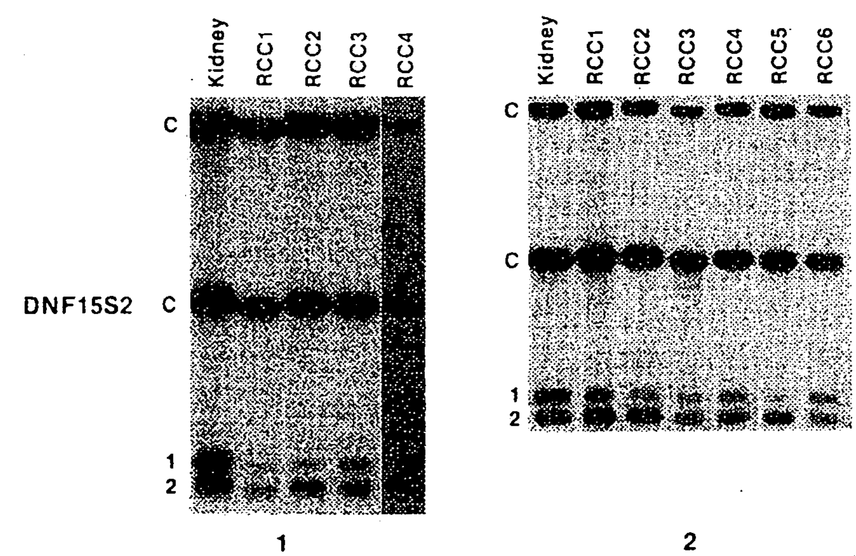


**Figure 1.** Analysis of alleles at a locus on chromosome 3p in renal cell carcinomas associated with von Hippel-Lindau disease. Panels show autoradiographs of Southern blot filters of DNA samples from renal cell carcinomas of two patients with von Hippel-Lindau disease. After bilateral nephrectomy, DNA was isolated from multiple, discrete renal cell carcinomas and corresponding normal kidney, digested with appropriate restriction enzymes, fractionated by agarose gel electrophoresis, and transferred to nylon filters. Filters were hybridized with radiolabeled probe pH3H2. Patient No. are shown below the autoradiographs. Numbers on the left of the panels indicate the observed alleles, with "1" and "2" referring to the larger and smaller allelic restriction fragments, respectively. "C" indicates a constant DNA fragment. In each patient, the DNA fragments in normal tissue were compared with those in the tumor. Kidney = DNA from kidney not involved with tumor; RCC1-RCC4 = individual RCC removed from the right and left kidneys of patient 1; RCC1-RCC6 = individual RCC removed from the right and left kidneys of patient 2.

Table 3. Parental origin of chromosome 3p alleles lost in tumors from patients with von Hippel-Lindau disease*

| Patient No. | Chromosome 3p locus | Affection status: Father's constitutional genotype | Mother's constitutional genotype | Patient's constitutional genotype | Patient's tumor genotype |
|---|---|---|---|---|---|
| 1 | DNF15S2 | 11(NA) | 12(A) | 12(A) | 2 |
| 3 | D3S32 | 12(A) | 22(NA) | 12(A) | 1 |
| | ERBAβ | 12(A) | 22(NA) | 12(A) | 1 |
| 7 | D3S32 | 12(NA) | 11(A) | 12(A) | 1 |

*Inheritance of chromosome 3p alleles lost (and retained) in tumors from patients with von Hippel-Lindau disease. "A" indicates an individual affected with von Hippel-Lindau disease; "NA" indicates an individual not affected with von Hippel-Lindau disease; "11" or "22" indicates that the individual was homozygous at that locus; "12" indicates that the individual was heterozygous at that locus. "1" indicates retention of the larger allele and loss of the smaller allele; "2" indicates retention of the smaller allele and loss of the larger allele. A number "1" or "2" with an underline indicates a chromosome 3p retained in the tumor and inherited from the patient affected with von Hippel-Lindau disease. Tumors analyzed were: patients 1 and 7, renal cell carcinoma; patient 3, pheochromocytoma. Conclusions as to origin of 3p alleles lost were reached as follows: Constitutional tissue of patient 1 was heterozygous (*12*) at the DNF15S2 locus. Patient 1 must have inherited allele 2 from his affected mother (genotype 12) and allele 1 from nonaffected father (genotype 11). Allele 1 *from the patient's father* was lost in the renal cell carcinoma.

loss at a locus (HBG) on chromosome 11p; the pheochromocytoma also had loss at a locus on chromosome 21. The high frequency of retention of heterozygosity indicates that the loss of chromosome 3p sequences in renal cell carcinomas of von Hippel-Lindau patients shows chromosomal specificity.

## Discussion

A unique feature of the present study was the analysis of multiple tumors that developed in individuals with an inherited predisposition to develop cancer. In familial renal cell carcinoma like sporadic renal cell carcinoma (*19*), we found a consistent loss of alleles at loci on the short arm of chromosome 3. In familial renal cell carcinoma there was a pattern to chromosome 3p allele loss. Multiple renal cell carcinomas in individual patients with von Hippel-Lindau disease showed loss of the same chromosome 3p alleles, which demonstrated that the same chromosome was deleted in each tumor. In all (five of five) hereditary renal cell carcinomas that could be evaluated for maternal or paternal origin of 3p alleles, the chromosome 3p alleles that were lost were located on the chromosome 3 bearing the wild-type allele of the VHL gene. These observations indicate that the loss of the balancing allele of the VHL gene is a prerequisite for tumorigenesis in renal cell carcinoma associated with von Hippel-Lindau disease.

Two other histologic types of tumors associated with von Hippel-Lindau disease were examined for chromosome 3p allele loss. The pheochromocytoma showed loss of the chromosome 3p bearing the wild-type allele of the VHL gene. Two hemangioblastomas from patient 5 showed loss of the same chromosome 3p alleles. These results suggest that the VHL gene, like the bilateral acoustic neurofibromatosis and retinoblastoma genes, is a recessive oncogene with pleiotropic action. Chromosome 3p allele loss observed in renal cell carcinoma, pheochromocytoma, and central nervous system hemangioblastomas associated with VHL represents the second step of a two-mutation process uncovering a null mutation at the VHL locus on chromosome 3p (*24,25*).

Hemangioblastomas are complex neoplasms containing at least two distinct histologic cell types. What remains unresolved is which cell type(s) is (are) neoplastic. This uncertainty makes it difficult for investigators to estimate accurately the proportion of neoplastic cells in hemangioblastomas and to correlate such estimates with changes in allele signal intensity. Our inability to detect chromosome 3p allele loss in the hemangioblastoma of patient 3 may reflect a high proportion of normal cells in these tumors, or alternatively, the presence of a small deletion. Simultaneous application of cell separation techniques and DNA polymorphism analysis to hemangioblastomas would be expected to improve detection of 3p deletion and to identify the cell type that contains this genetic change.

The VHL gene product appears essential for the maintenance of normal differentiation of several distinct cell types. Formation of cysts and aberrant blood vessels are characteristic of aberrant differentiation in tumors associated with von Hippel-Lindau disease. Isola-

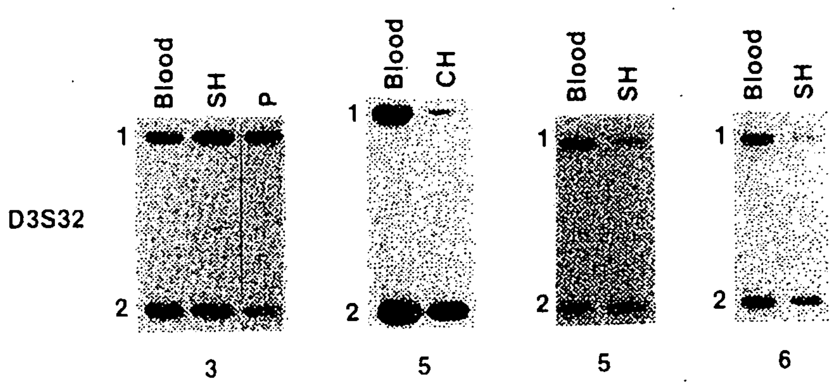


Figure 2. Analysis of alleles at a locus on chromosome 3p in hemangioblastomas and a pheochromocytoma associated with von Hippel-Lindau disease. DNA from tumor samples and corresponding peripheral blood leukocytes were digested with appropriate restriction endonucleases and analyzed by the Southern blot technique. Filters were hybridized with radiolabeled probe pEFD145.1 (D3S32). Patient No. are shown below the panels. Numbers on the left indicate the observed alleles with "1" and "2" referring to the larger and smaller allelic fragments, respectively. Blood = DNA from peripheral blood leukocytes; P = DNA from a pheochromocytoma; SH = DNA from spinal hemangioblastomas; CH = DNA from a cerebellar hemangioblastoma.

Table 4. Specificity of chromosome allele loss in patients with von Hippel-Lindau disease*

| Patient No. | Tumor | Chromosome | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 2 D2S44 (M) | 2 D2S44 (T) | 4 D4S125 (R) | 6 D6S37 (H) | 11 HBG (H) | 11 INS (P) | 13 D13S1 (M) | 15 D15S1 (M) | 16 HP2 (H) | 16 D16S83 (R) | 17 D17S4 (T) | 17 D17S5 (T) | 21 D21S113 (M) |
| 1 | RCC1 | 12 | | 12 | 12 | 12 | | — | 12 | 12 | — | | 12 | 12 |
| | RCC2 | 12 | | 12 | 12 | 1 | | — | 12 | 12 | — | 12 | 12 | 12 |
| | RCC3 | 12 | | 12 | 12 | 12 | | — | 12 | 12 | — | 12 | 12 | 12 |
| | RCC4 | 12 | | | | 12 | | — | | | — | | | |
| 2 | RCC1 | | 12 | — | — | | 12 | 12 | 12 | | 12 | 12 | 12 | 12 |
| | RCC3 | | 12 | — | — | | 12 | 12 | 12 | | 12 | 12 | 12 | 12 |
| | RCC5 | | 12 | — | — | | 12 | 12 | 12 | | | 12 | 12 | 12 |
| | RCC6 | | 12 | — | — | | 12 | 12 | 12 | | | 12 | 12 | 12 |
| 3 | P | 12 | | — | — | 1 | | — | 12 | | 12 | 12 | 12 | 1 |

*Specificity of chromosome 3p loss in tumors from patients with von Hippel-Lindau disease. *See* table 1 footnote for description of symbols. Letters in parentheses indicate the restriction endonuclease: T, *Taq*I; M, *Msp*I; H, *Hin*dIII; R, *Rsa*I; P, *Pvu*II.

tion of the VHL gene will be invaluable in the development of predictive tests and in comprehension of the pathogenesis of this disease.

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